0888-8809/05/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 19(10):2478–2490

doi: 10.1210/me.2005-0072

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# Development of Peptide Antagonists for the Androgen Receptor Using Combinatorial Peptide Phage Display

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**Under the auspices of the Nuclear Receptor Signaling Atlas (NURSA), we have undertaken to evaluate the feasibility of targeting nuclear receptor-coactivator surfaces for new drug discovery. The underlying objective of this approach is to provide the research community with reagents that can be used to modulate the transcriptional activity of nuclear receptors. Using combinatorial peptide phage display, we have been able to develop peptide antagonists that target specific nuclear receptor (NR)-coactivator binding surfaces. It can be appreciated that reagents of this nature will be of use in the study of orphan nuclear receptors for whom classical ligands have not yet been identified. In addition, because the interaction of coactivators with the receptor is an obligate step for NR transcriptional activity, it is anticipated that peptides that block these interactions will enable the definition of the biological and pharmacological significance of individual NR-coactivator interactions. In this report, we describe the use of this approach to develop antagonists of the androgen receptor by targeting its coactivator-binding pocket and their use to study the coactivator-binding surface of this receptor. Based on our findings, we believe that molecules that function by disrupting the androgen receptor-cofactor interactions will have use in the treatment of prostate cancer. (*Molecular Endocrinology* 19: 2478–2490, 2005)**

THE NUCLEAR RECEPTOR (NR) superfamily of transcription factors are involved in multiple physiological processes ranging from sexual development to energy homeostasis and fat metabolism (1). Despite the functional diversity exhibited by this class of transcription factors, they share a remarkable structural and functional similarity. In the absence of ligand, they exist within target cells in a transcriptionally inactive form. Upon binding ligand, they undergo a conformational change that initiates a cascade of events, ultimately leading to their association with specific DNA sequences within target gene promoters. The magnitude of the resulting response is influenced by both the promoter context and the nature and abundance of the cofactors required for receptor transcriptional activity. Structural studies have revealed that the ligand binding domains (LBDs) of most NRs are comprised of 12 antiparallel helices that undergo significant rearrangement upon agonist binding. This movement creates a shallow hydrophobic groove atop the ligand binding pocket, allowing LxxLL motif-containing coactivators to dock (2–4). This hydrophobic groove, generally referred to as the "coactivator binding pocket" or "activation function 2" (AF-2), is apparent in the crystal structures of all of the nuclear receptors that have been studied thus far and is considered to be the major protein-protein interaction site on the surface of the NR-LBD (5). It is not surprising, therefore, that the isolated LBD from most NRs, when tethered to DNA, exhibits autonomous transcriptional activity because this domain contains all that is required to enable the recruitment of the key LxxLL-containing coactivators such as steroid receptor coactivator-1, 2, and/or 3 (p160 coactivators) (6–11). Because this general mechanism of coactivator recruitment appears to be used by most members of this receptor superfamily and the coactivator recruitment is an obligate step in NR action, it is not surprising that disruption of this receptor:cofactor interface inhibits the transcriptional activity of most receptors. Traditionally, transcriptional inhibition is achieved using compounds that competitively inhibit agonist binding and allow the receptor to adopt a conformation that is incompatible with coactivator recruitment. However, as the field has advanced it has become apparent that pharmacological tools, other than competitive antagonists, are needed

**First Published Online July 28, 2005**

Abbreviations: AF, Activation function; AR, androgen receptor; Asc-1, activating signal cointegrator 1; BirA, *Escherichia coli* biotin holoenzyme synthetase; bt-AR, avitag-AR; casx, casodex; DBD, DNA binding domain; DHT, dihydrotestosterone; ER, estrogen receptor; GR, glucocorticoid receptor; LBD, ligand binding domain; M2H, mammalian two-hybrid; MMTV-Luc, mouse mammary tumor virus-luciferase; NR, nuclear receptor; NURSA, Nuclear Receptor Signaling Atlas; OH-F, hydroxyflutamide; PR, progesterone receptor; RTI, Research Triangle Institute; SARM, selective AR modulator; TRRAP, transformation/transcription-associated protein; VP1b, herpes simplex virus VP16 transactivator protein; wt-AR, wild-type AR.

***Molecular Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

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to probe the signaling networks in which the NRs participate. This realization has come from the discovery of 1) orphan NRs that recruit coactivators in a constitutive manner in the absence of an apparent ligand, 2) signaling pathways that impinge on NRs obviating the need for ligand, and 3) surfaces that interact with coactivators whose presentation are not disrupted by available antagonists (12–16). Reflecting the evolving complexity of the NR signaling pathway(s) we have undertaken, as our contribution to the Nuclear Receptor Signaling Atlas (NURSA) initiative, to use combinatorial peptide phage display to develop peptide antagonists that could be used as tools to define the protein-protein interaction surfaces on each of the NRs that are important for transcriptional activity and that may serve as targets for new drug discovery. In this paper, we describe the use of this approach to study the androgen receptor (AR) signal transduction pathway.

Although the general mechanism of action of AR is similar to other members of the NR superfamily, there are significant differences that have hindered our understanding of antiandrogen action and have limited the success of finding new antagonists of this receptor. Specifically, it is now known that the canonical AF-2 pocket in AR is used primarily to accommodate an intermolecular association of the amino terminus of the receptor with the carboxyl terminus and is required for high-affinity androgen binding (17–19). However, it does not contribute directly to the transcriptional activity of the full-length receptor because 1) the isolated AF-2 alone has negligible activity (17), 2) deletion of the ligand-binding domain of AR has minimal impact on its transcriptional activity (20), 3) synthetic androgens that do not facilitate N- and C-terminal interactions can activate some AR-responsive genes (21), and 4) peptides that block N- and C-terminal interactions do not inhibit AR transcriptional activity (22). We infer from these results that the region defined as AF-2 within NRs has a unique role in AR function and that additional surfaces required for coactivator interaction remain to be defined. By extension it is likely that most of the well-studied coactivators, such as those of the p160 class, are recruited to AR in an AF-2-independent manner. Indeed, the obvious mechanistic differences between AR and other closely related members of the nuclear receptor superfamily make it likely that the coactivators used by AR and the surfaces on the receptor with which they interact are likely to be unique.

Given the unique nature of the AR signal transduction pathway, we have chosen as part of our contribution to the overall goals of the NURSA, to use combinatorial peptide phage display to 1) define the coactivator binding surfaces on AR, 2) develop high-affinity peptide antagonists that block receptor-cofactor interactions, and 3) use the sequences derived from the interacting peptides to identify AR-interacting proteins *in silico*. The results of these efforts have yielded tools with which to study AR action and have revealed that the coactivator transformation/transcription domain-associated protein (TRRAP) is a candidate regulator of AR pharmacology. The general approach used in this project has been applied to other receptors with the goal of complementing other proteomic/genomic initiatives ongoing under the auspices of NURSA.

## RESULTS

### Selection of High-Affinity AR Binding Peptides Using Combinatorial Phage Display

Previously we have used combinatorial phage display to identify LxxLL-containing peptides that interact with the AF-2 domain of the estrogen receptor (ER) and effectively inhibit estradiol-mediated transcriptional activation when introduced into cells (16, 23, 24). Interestingly, a subset of these peptides were shown to interact very well with agonist-activated AR, but surprisingly they had no effect on androgen agonist activity when expressed in target cells (22). This suggested either that the peptides were not able to compete with coactivators for AF-2 binding or that this domain was not critical for transcriptional activation. This encouraged us to perform a new primary phage display screen to identify peptides that could interact with purified full-length AR with high affinity and which may be enlightening with respect to the coactivator binding surfaces on this receptor.

For these studies, we chose to use full-length receptor because previous studies we have performed with other receptors have indicated that the conformation of the coactivator-binding pocket within the context of the isolated LBD and the full-length receptor are not the same (25). In the past, this type of study with AR was precluded by the difficulty of producing soluble, biologically active full-length receptor. However, using the recently described BioBac expression system, we have successfully overproduced full-length biologically active AR in insect cells (Fig. 1) (26). In short, we have introduced an Avitag (a 22-amino acid in-frame sequence) at the amino terminus of AR that contains a canonical biotinylation sequence for the *Escherichia coli* biotin holoenzyme synthetase (BirA) (27). We confirmed that addition of the tag did not affect the transcriptional properties of the receptor by demonstrating that its transcriptional activity was comparable to wild-type AR (wt-AR) in a cotransfection assay in CV-1 cells (Fig. 1A). The modified AR cDNA was then cloned into a baculovirus expression vector and introduced into insect cells together with an expression vector for the BirA enzyme. The recombinant receptor was then purified to homogeneity using streptavidin affinity chromatography (Fig. 1B). Using this enabling reagent, we proceeded to screen for AR-interacting peptides using M13 phage display with the anticipation that we may identify cofactor binding sites located outside of the LBD or sites within this

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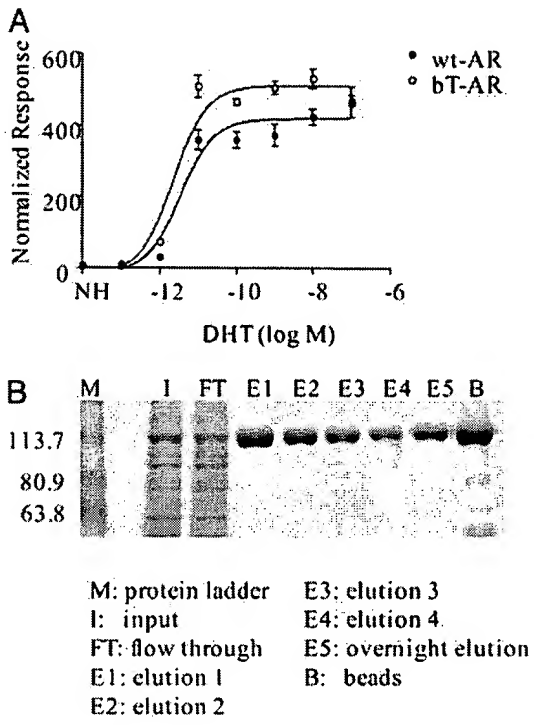


**Fig. 1.** Production and Purification of Full-Length AR Using the BioBac System

A, The activity of biotin acceptor peptide-tagged AR was assessed in transient transfection assay. CV-1 cells were transfected with either the wt-AR or the bT-AR, together with an expression plasmid for BirA enzyme, an AR-responsive MMTV-luc reporter gene, and a normalization plasmid pCMV$\beta$gal. Different concentrations of the AR ligand DHT were added to cells 24 h after transfection, and the luciferase and $\beta$-galactosidase activities were measured 16 h after ligand addition. The luciferase activity was normalized to those of the $\beta$-galactosidase and expressed as normalized response. B, bT-AR was purified to greater than 95% homogeneity using monomeric-avidin column. Protein fractions collected from column purification were resolved on a 7.5% SDS-PAGE, fixed and visualized with Coomassie staining.

domain that require the amino terminus for full function.

We have previously published detailed protocols describing combinatorial peptide phage display and the construction of the random libraries and only the specifics related to AR will be expanded upon (28, 29). Specifically, R1881-activated AR was immobilized directly on a 96-well plastic plate in the presence of 0.1 M $NaHCO_3$ buffer. To each well was then added approximately $10^8$ phage from each of five different M13 phage display libraries. The formats of the libraries used in our screens are listed in Fig. 2, including one expressing conformationally constrained cyclic peptides, two 13-oligomer peptide libraries, one with a fixed histidine and another with a fixed tyrosine placed at the center of the peptide, and an $X_7$LxxLL$X_7$ domain library we have used previously to identify peptide antagonists for other NRs (24, 29–31). After enrichment of AR-binding phage, we shotgun subcloned the DNA inserts corresponding to the recombinant peptides directly into a mammalian two-hybrid (M2H) vector, allowing the peptides to be expressed as fusions with the Gal4 DNA binding domain (DBD). The interactions of these peptides with the full-length AR were confirmed in a M2H assay (not shown), and all of the peptides that scored positive in this assay were sequenced (Fig. 2). The corresponding FxxLF motifs from ARA54 and the amino terminus of AR are included for comparative purpose. Surprisingly, all of the peptides, regardless of which libraries from which they were originally derived, contain a phenylalanine-rich, FxxLF-like motif (Fig. 2). Interestingly, we recovered only two peptides, out of $10^8$ possible different phage from the LxxLL library, and both of these peptides contain a phenylalanine-rich motif embedded within their sequences. This is in stark contrast to the screens we have performed previously with other NRs where diverse LxxLL containing motifs were enriched. However, this result is in good agreement with findings of others that indicated that the protein-protein interaction surface(s) on AR does not favor the binding of LxxLL containing peptides (22, 32, 33).

One of the primary goals of this project was to define the coactivator pockets on AR and use this information to develop peptide antagonists that can be used to probe different AR-cofactor interactions. In this regard, it was important to understand the degree to which the AR-interacting peptides interacted with other nuclear receptors. This was accomplished using a M2H, where the ability of the selected Gal4-peptide fusions to recruit full-length glucocorticoid receptor (GR), full-length progesterone receptor B (PR-B), and full-length ER-$\alpha$ (ER$\alpha$), those most related to AR, were assessed (Fig. 2 and supplemental data published on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). Notably, none of the peptides showed significant interaction with GR or ER$\alpha$, indicating that the AF-2 pocket on AR is significantly different from those of the GR and ER. We were surprised to find, however, that a number of peptides, 532-1 and 822-2 for example, interacted very well with PR-B. This result suggested that the structure of the PR AF-2 may be more similar to AR, allowing it to accommodate both LxxLL and FxxLF motifs. The physiological significance of this apparent structural similarity will be explored in follow-up projects.

We next performed a detailed comparison of the AR-interaction properties of the isolated peptides and how they differed from the FxxLF motif ($^{23}$FQNLF$^{27}$) found within the amino terminus of AR that also interacts with AR AF-2. Firstly, we used the M2H assay to analyze the binding characteristics of the identified peptides in the presence of a pure agonist dihydrotestosterone (DHT), two antagonists, casodex (casx) and hydroxyflutamide (OH-F), as well as two selective AR modulators (SARMs), Research Triangle Institute (RTI)-001 and RTI-018 (Fig. 3). The latter two compounds, when bound to AR, induce a conformational change that prevents the association of the amino and

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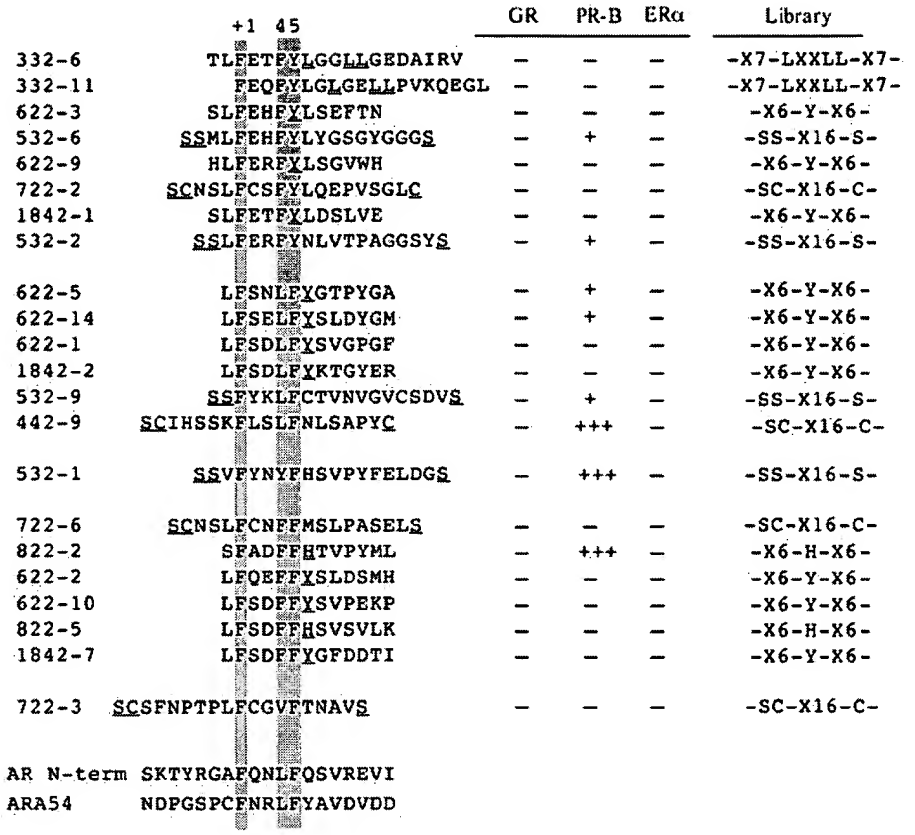


**Fig. 2.** All the AR-Binding Peptides Contain Variations of the FxxLF Motif and Most of Them Do Not Cross-React with Other Homologous Receptors

The sequences of peptides identified in M13 peptide display are aligned based on the phenylalanine-rich motif. Fixed residues in the libraries are *underlined.* The corresponding FxxLF motifs from ARA54 and the amino terminus of AR are included for comparative purposes. Mammalian two-hybrid assay was used to determine the interactions of these peptides with receptors indicated. Fold interaction was calculated by dividing the normalized luciferase responses produced in the presence of indicated VP16-receptor to those of the VP16 alone. –, Less than 10-fold interaction. +, Interaction between 10- and 50-fold. ++, 50- to 100-fold. +++, Interaction of greater than 100-fold (for details see supplemental data). Formats of peptide libraries used in the screen. X , Any amino acid; *numbers next to the X* denote the number of amino acids, *i.e.* X6: six random amino acids.

carboxyl termini of the receptor (21). The results of this analysis indicate that the $^{23}$FQNLF$^{27}$ motif interacted strongly with the agonist DHT-activated AR, although a weaker interaction was observed in the presence of the compound RTI-018. No interaction was observed when cells were treated with bicalutamide, flutamide, or RTI-001. This interaction pattern is in good agreement with previous findings in that the N- and C-terminal interaction appears to correlate with the agonist activity of AR ligands (17–19, 34). When we tested the peptides identified from our screens in a similar manner we were surprised to find that even though they shared a considerable degree of sequence similarity, they interacted with AR in a manner distinct from the $^{23}$FQNLF$^{27}$ motif. Specifically, we observed that most of the peptides identified bind to AR in the absence of ligand, and are not displaced from the receptor in the presence of the antagonists casodex or flutamide or the SARMs RTI-018 and RTI-001 (Fig. 3). Thus, although all of the interacting peptides contain the core sequence FxxLF, they appear to interact with AR in a manner distinct from the $^{23}$FQNLF$^{27}$ motif contained within the AR amino terminus. In support of this contention, we have observed that deletion of helix 12 from the receptor reduces, but does not obliterate, the interaction of the isolated peptides with receptor activated by the SARMs RTI-018 and RTI-001 (data not shown). We have also demonstrated (not shown) that the AR-LBD is necessary and sufficient for peptide interaction, a result we find surprising given the fact that their interaction with AR is not influenced by the nature of the bound ligand. As will be discussed below, this finding may have significance with respect to the development of antiandrogen resistance in prostate cancer.

### Development of Phenylalanine-Rich Peptides as Potent AR Antagonists

It has been determined from work performed in our laboratory and that of others that combinatorial phage display using protein targets usually leads to the iden-

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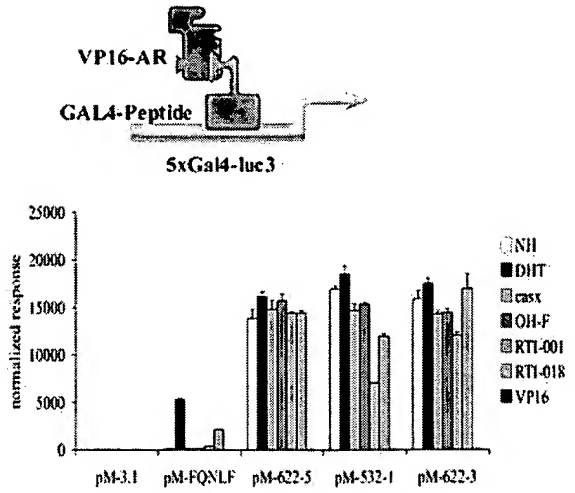


**Fig. 3.** A Mammalian Two-Hybrid Assay Was Used to Confirm the Interaction of Peptides with Full-Length AR in Cells

HepG2 cells seeded in 24-well plates were transfected with Gal4DBD-peptide (108 ng/well) and VP16-AR (108 ng/well), together with a reporter construct 5xGal4-Luc3 (567 ng/well) and a normalization plasmid pCMVβgal (27 ng/well). Different AR ligands at 100 nM concentration were added 24 h after transfection and the luciferase and β-galactosidase activities were measured 16 h after ligand addition. The values of the luciferase activity were normalized to those of the β-galactosidase and expressed as normalized response. NH, Vehicle control (100% ethanol); VP16, empty VP16 vector was used instead of VP16-AR. All hormones were used at saturating concentration (100 nM).

tification of peptides that interact with *bona fide* protein-protein interaction surfaces (35, 36). Thus, we next tested the functionality of the surfaces on AR with which the identified peptides interacted. Although AR action in cells is likely to be a composite of both its genomic and nongenomic actions, we chose in these initial studies to evaluate the effects of the peptides on androgen-mediated transcriptional activity. For these studies, Gal4-peptide fusions were expressed in cells and their ability to inhibit AR-mediated transcription in CV-1 cells expressing exogenous receptor and different AR-responsive reporter genes was measured. As shown in Fig. 4A, the fusions pM-622-5 and pM-532-1 were able to completely inhibit AR transcriptional activity, whereas a fusion expressing the peptide pM-FQNLF (derived from the AR amino terminus) was unable to inhibit transcription of the receptor. Similar findings were obtained when we evaluated the ability of the fusions to inhibit the partial agonist activity of the SARM RTI-018 (Fig. 4B). All fusions were expressed in cells at comparable levels and had no effect on AR expression levels in cells (not shown). Cumulatively, the results of this analysis revealed that both pM622-5 and pM-532-1 target a surface on AR that is absolutely required for transcriptional activity. Previously, we had shown that LxxLL-containing peptides when expressed as Gal-4 fusions were able to disrupt AR-N- and C-terminal interactions (22). However, rather than inhibiting AR transcriptional activity they were shown to mimic the stabilizing interaction of the amino terminus with AF-2 and facilitate ligand binding. We believe, based on our new data, that the $^{23}$FQNLF$^{27}$ fusion probably functions in a similar manner as the previously studied LxxLLs. The peptides pM-622-5 and pM532-1, however, distinguish themselves from other AR-interacting peptides because they effectively inhibit androgen-dependent transcriptional activity. In addition, they inhibit the partial agonist activity of RTI-018, a compound that does not facilitate or require N- and C-terminal interactions for activity (Fig. 4B). We conclude that these peptides inhibit a fundamental aspect of AR signaling that may result from their ability to interact with the AF-2 domain of this receptor. In support of this hypothesis, we were able to show that overexpression of these peptides interferes with the ability of ARA54, an AR AF-2 coactivator, to potentiate AR transcriptional activity (Fig. 4C). The precise surface they occupy will require further investigation by mutagenesis or crystallography.

We next wished to determine whether the inhibitory effect of these peptides is specific to AR. This analysis was performed in CV-1 cells after transfection of the mouse mammary tumor virus-luciferase (MMTV-Luc) reporter gene (activated by either GR, PR, or AR) and either the AR (Fig. 5A) or PR-B (Fig. 5B) expression vectors. As observed above, DHT-mediated activation of MMTV-Luc transcriptional activity was inhibited by either of three different AR-selective peptides (pM-332-6, pM-532-1 or pM-622-3). However, a peptide that is highly selective for PR and GR, Lx23 (Kimbrel, E. A., and D. P. McDonnell, unpublished result), had no effect on AR transcriptional activity (Fig. 5A). When an analogous experiment was performed to examine the effects of these peptides on PR transcriptional activity we observed, not surprisingly, that pM-Lx23 efficiently inhibited R5020-activated PR transcriptional activity. Expression of either pM-332-6 or pM-622-3 had no impact on PR transcription (Fig. 5B). However, a slight inhibition of PR activity by pM-532-1 was observed, reflecting the positive interaction of this peptide with PR noted above. These data indicate that the AR-interacting peptides identified in this screen were capable of functioning as peptide antagonists of AR transcriptional activity by interfering with an obligate step in receptor signaling.

### FxxLF-Containing AR-Interacting Peptides Can Be Separated into Two Functionally Distinct Groups

A recent study by Hur *et al.* (37) described the identification of peptides (EH series by our nomenclature) that interacted with AR in a ligand-dependent manner. A significant difference between their screening methodology and ours is their use of the isolated LBD as bait for combinatorial peptide phage display rather than the intact receptor. Not surprisingly, most of the peptides isolated with this approach were subse-

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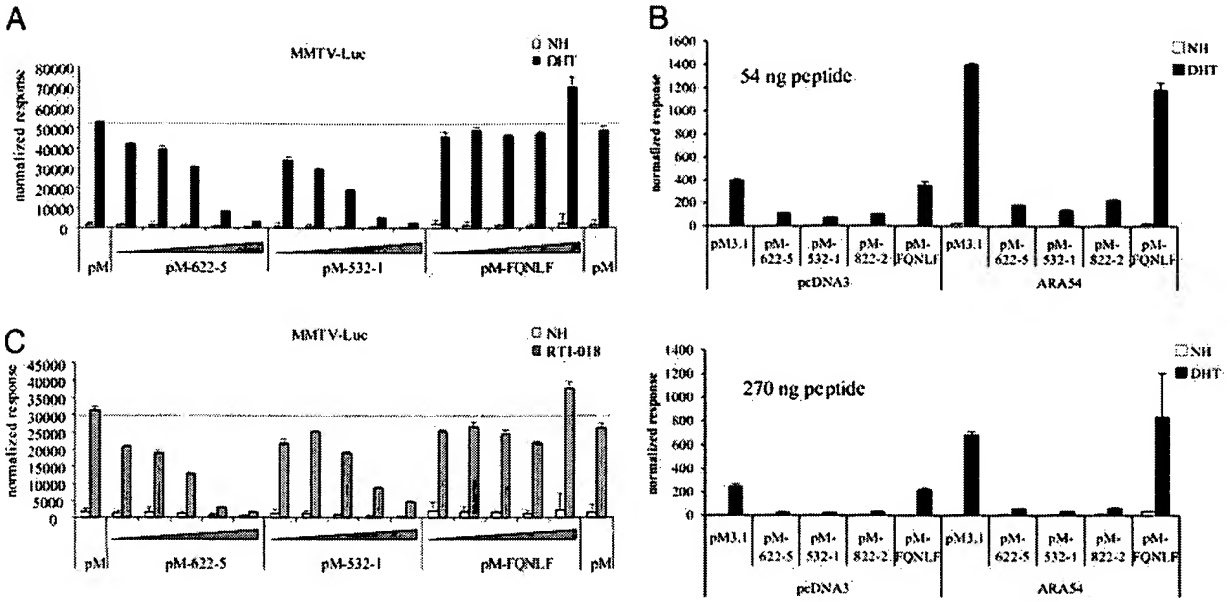


**Fig. 4.** AR-Interacting Peptides Are Potent Inhibitors of AR Transcriptional Activity

CV-1 cells seeded in 24-well plates were transfected with increasing amounts of Gal4DBD-peptide expressing constructs (ranging from 13.5–270 ng/well), together with an AR expression plasmid pSG5-AR (13.5 ng/well), an AR-responsive MMTV-luc reporter gene (500 ng/well), and a normalization plasmid pCMV$\beta$gal (27 ng/well). The pM serves as a negative control containing only the Gal4DBD without peptide. Empty pM was also used to adjust input DNA amounts so that all the wells were transfected with equal amount of pM containing plasmid DNA. Vehicle control, AR ligands DHT, 10 nM (A) and RTI-018, 100 nM (B) were added to cells 24 h after transfection and the luciferase and $\beta$-galactosidase activities were measured 16 h after ligand addition. The values of the luciferase activity were normalized to those of the $\beta$-galactosidase and expressed as normalized response. C, CV-1 cells were transfected with either pcDNA3 empty vector (214 ng/well) or pcDNA3-ARA54 (270 ng) together with pSG5-AR (13.5 ng/well), MMTV-luc reporter gene (500 ng/well), and pCMV$\beta$gal (27 ng/well). The ability of peptides to inhibit the ability of ARA54 to potentiate AR was tested by cotransfecting two doses of various peptides (54 and 270 ng) as indicated. The amounts of DNA transfected in each well were adjusted to a total of 810 ng/well with pBlueScript plasmid.

quently shown by crystallography to interact with AR in a manner similar to $^{23}$FQNLF$^{27}$. The peptides identified in our screens using full-length receptor were functionally distinguishable from the later FxxLL-containing peptide. Given the information provided by the crystal structures described by Hur *et al.*, it was important to determine whether the EH series of peptides could function as peptide antagonists. If so, then the structural information in these latter studies would have a significant impact on our ability to optimize peptide antagonists. For these studies we initially examined the ability of each of the EH peptides, as a fusion to the Gal4-DBD, to interact with AR in the presence of various ligands using a M2H assay. The results of this analysis, shown in Fig. 6A, indicate that the AR-interacting profile of all but one of the EH peptides was similar to the $^{23}$FQNLF$^{27}$ in that they interacted with AR in the presence of agonists but not antagonists. However, one peptide, pM-EH5, was shown to display pharmacological properties similar to the selective peptides we identified using full-length AR, suggesting that it was distinct from those peptides that merely mimicked the amino terminal $^{23}$FQNLF$^{27}$ motif. We did not detect significant interaction between AR and two of the peptides, pM-EH3 and pM-EH4, likely due to their weak affinity for the receptor (data not shown). Our studies detailed above indicated that peptides that interacted with AR in a manner similar to the pM-EH5 should function as antagonists of AR signaling. To test this hypothesis, we compared the antagonist properties of pM-622-5, pM-532-1, pM-$^{23}$FQNLF$^{27}$ and each of the EH series peptides in CV-1 cells using a transfected MMTV-luc reporter. As shown in Fig. 6B, all the EH peptides tested were expressed at the same level, yet they had dramatically different activities as antagonists. From these studies, the most interesting result is that pM-EH5, the only EH series peptide that distinguished itself from $^{23}$FQNLF$^{27}$ functioned as an effective AR antagonist. These findings support our hypothesis that AR-interacting FxxLF-containing peptides fall into two classes: 1) those that functionally resemble the natural FxxLF in the amino terminus and that do not have significant antagonist properties; and 2) those that are more like pM-622-5 or pM-532-1, which interact with AR in the presence of any ligand and function as effective antagonists. Given the fact that EH5 has been shown by crystallography to interact with the AR-AF-2 domain, it is likely that both classes of peptides interact with the same domain but in a different manner.

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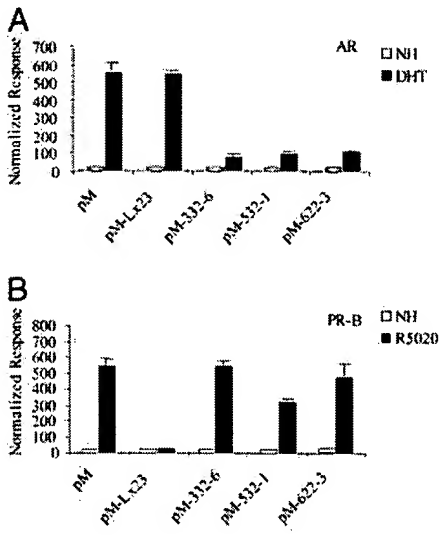


**Fig. 5.** AR-Interacting Peptides Selectively Inhibited AR But Not PR-Mediated Reporter Gene Activation

CV-1 cells were transfected with expression plasmids for GalDBD-peptide (54 ng/well) as indicated, together with a reporter gene MMTV-Luc (229.5 ng/well), a normalization plasmid pCMV$\beta$gal (27 ng/well), and either an AR expression plasmid SG5-AR (13.5 ng/well) (A) or a PR expression plasmid pcDNA3-PRB (13.5 ng/well) (B). Agonists, DHT (10 nM) for AR and R5020 (10 nM) for PR were added 24 h after transfection. Luciferase and $\beta$-galactosidase activities were measured 16 h after ligand addition. The values of the luciferase activity were normalized to those of the $\beta$-galactosidase and expressed as normalized response.

### Identification of AR-Interacting Proteins *in Silico*

One of the most interesting, though predictable, aspects of combinatorial phage display is that the peptides isolated usually share a high degree of sequence homology to domains in proteins that are known partners of target proteins (35, 38). This observation means that this technology can be used to effectively identify peptide motifs required for specific protein-protein interactions. However, even more powerful is the ability to use the consensus sequence(s) derived from groups of target-interacting peptides to identify corresponding proteins by appropriate database searches. We have previously used the latter approach to successfully identify protein partners of the nuclear receptor corepressor (NCoR) (36). It is likely that the peptides identified in our screens mimic the binding domains of AR-interacting proteins rather than the amino terminal $^{23}$FQNLF$^{27}$ motif. Thus, we searched both protein and nucleic acid databases for proteins homologous to the peptides identified in our screens (Fig. 7). Using the sequence 822-2, we identified the 400-kDa coactivator protein TRRAP (PAF400) as a potential AR-interacting protein. TRRAP is a component of the TFTC complex (TATA-binding protein-free TAF-containing complex) and was previously characterized as a coactivator for ER$\alpha$ and p53 (39–41). Searches using peptides 332-6 and 532-6 both identified the 50-kDa subunit (p50) of the Asc-1 (activating signal cointegrator 1) complex as a potential AR interactor (42, 43). Interestingly, the Asc-1 itself has been shown to interact with the hinge region of AR and function as an AR coactivator (44). It is possible that AR may use distinct domains to contact both p50 and Asc-1 in the same protein complex, and the two cofactor proteins then act in concert to modulate AR function. Additionally, the peptide 532-6 also shared a high degree of homology with the extreme C terminus of the splice isoform a of the histone deacetylase 9 (45). To probe the significance of the factors identified, we first performed a M2H assay to confirm that the region within the protein identified, homologous to the search object, was able to interact with AR. To this end, we cloned the appropriate fragments from each protein identified into the Gal4-DBD expression vector and tested their interaction with AR using a M2H assay. Notably, the p50 and TRRAP-derived peptides, but not the peptide derived from histone deacetylase 9, interacted with AR in the M2H assays (Fig. 7B). Although we will follow-up on these important findings in subsequent studies, we performed a preliminary investigation of the impact of manipulating TRRAP protein expression levels on AR biology to highlight the utility of this *in silico* approach to identify NR-interacting proteins.

The AR coactivation studies were performed by examining AR transcriptional activity on four different promoters in CV-1 cells in the presence or absence of ligands as indicated (Fig. 8). On all promoters, we observe that increasing input concentrations of the TRRAP expression vector led to an increase in DHT activation. The efficacy of the coactivator ranged between 4- and 10-fold depending on the promoter and was maximal at the highest input concentration of expression vector. The significance of this potentiation is highlighted by the fact that we were only able to achieve a modest (less than 2-fold) overexpression of TRRAP, as determined by Western immunoblot, under any conditions examined (data not shown). A similar effect of TRRAP on the partial agonist activity of the SARM RTI-001 was observed albeit only at the highest level of input expression vector. Interestingly, although TRRAP was identified by its homology to a peptide that could interact with both agonists and antagonists, it had no significant effect on the pharmacological properties of casodex. Beyond the scope of this study, we will continue to probe the significance of the AR-TRAAP interaction and the functional significance of other proteins identified by this *in silico* approach. However, the results presented clearly demonstrate the utility of using phage display as a means to identify novel AR-interacting proteins.

## DISCUSSION

Improved efforts at diagnosing prostate cancer at earlier stages have resulted in more patients presenting

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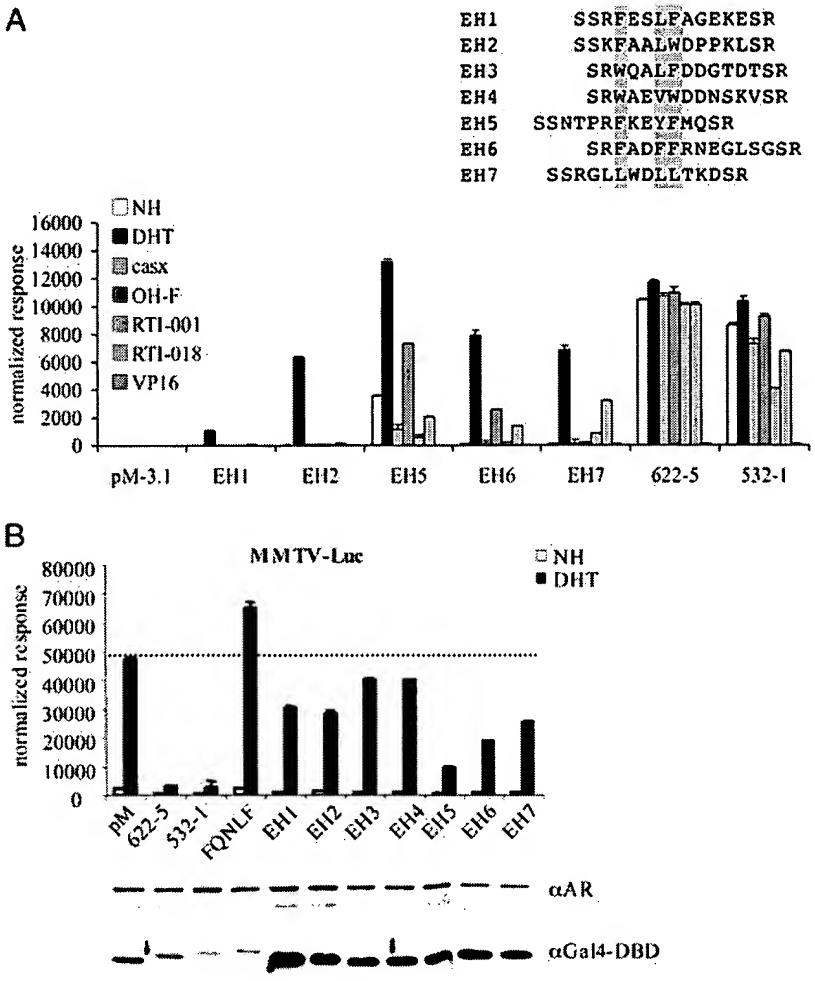


**Fig. 6.** Mammalian Two-Hybrid Assay Was Used to Analyze Interactions between AR and the EH Series of Peptides (37)

A, HepG2 cells were transfected with Gal4DBD-peptide (108 ng/well) and VP16-AR (108 ng/well), together with a reporter construct 5xGal4-Luc3 (567 ng/well) and a normalization plasmid pCMVβgal (27 ng/well). Different AR ligands (100 nM) were added 24 h after transfection and the luciferase and β-galactosidase activities were measured 16 h after ligand addition. Luciferase activity was normalized to that of β-galactosidase and expressed as normalized response. NH, Vehicle control; VP16, empty VP16 vector was used instead of VP16-AR. B, Comparison of the efficacies of various phenylalanine-rich peptide antagonists. CV-1 cells were transfected with an AR expression plasmid SG5-AR (13.5 ng/well) together with various Gal4DBD-peptide (270 ng/well) expression constructs as indicated, and a reporter construct MMTV-Luc (499.5 ng/well). A normalization plasmid pCMVβgal (27 ng/well) was also included in the transfection to normalize for transfection efficiency. Vehicle control (NH) or DHT was added 24 h after transfection and the luciferase and β-galactosidase activities were measured 16 h after ligand addition. The values of the luciferase activity were normalized to those of the β-galactosidase and expressed as normalized response. Expression of Gal4DBD-peptide and AR was detected with an anti-Gal4DBD antibody (Santa Cruz, sc-577) and an anti-AR 441 antibody, respectively.

with curable disease. Not surprisingly, therefore, the 10-yr survival rate has improved to over 70% for patients with organ-confined prostate cancer who are treated with radical prostatectomy or radiotherapy (46–48). However, despite the encouraging survival trend, prostate cancer remains the second most common cause of cancer death among men (49). For patients with cancer that has spread to distant organs, the disease remains incurable. Because prostate cancer cell proliferation can be stimulated by androgens via activation of AR, androgen deprivation, achieved either by inhibition of androgen synthesis or by using AR antagonists to block receptor activation, is commonly used in patients with recurrent or advance disease (50). Although effective initially, the responsiveness of cancer cells to these therapies is not robust, and the disease eventually advances to a hormone refractory state. Currently, the treatment options for androgen refractory disease are very limited and significant progress in the development of pharmaceutical interventions for this stage of the disease is hampered by the lack of a clear understanding of the molecular mechanism(s) by which resistance to hormonal therapies arises. What is becoming clear, however, is that although tumor growth resumes in the absence of androgens it appears to require AR (51).

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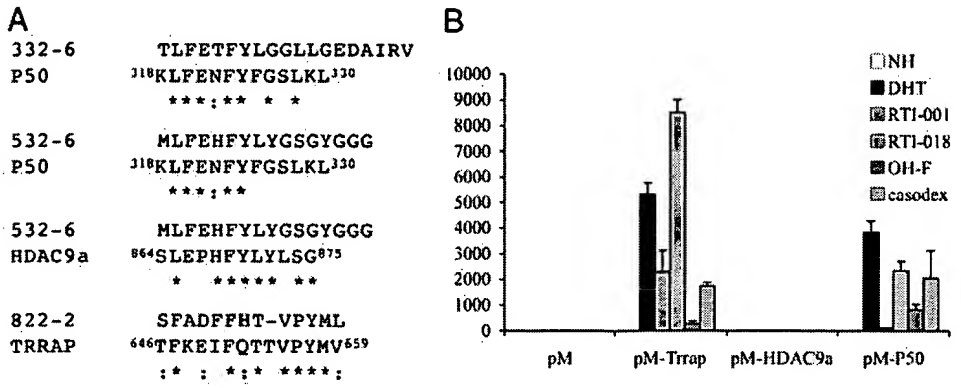


**Fig. 7.** Identification of Potential AR-Interacting Proteins *in Silico*

A, Peptide sequences and their corresponding protein fragments were aligned using MacVector ClustalW pairwise algorithm. Identical residues are labeled with an *asterisk (*) underneath*; and amino acids of similar chemical types are labeled with a *colon* (:). B, Peptides derived from candidate cofactors were subcloned into the Gal4DBD expression vector and their interaction with VP16-AR was analyzed in a mammalian two-hybrid assay. HepG2 cells were transfected with Gal4DBD-peptide (108 ng/well) and VP16-AR (108 ng/well), together with a reporter construct 5xGal4-Luc3 (567 ng/well) and a normalization plasmid pCMVβgal (27 ng/well). Different AR ligands at $10^{-7}$ M concentration were added 24 h after transfection and the luciferase and β-galactosidase activities were measured 16 h after ligand addition.

There is now strong consensus that the transcriptional activity of AR can be regulated in a ligand-independent manner. This pioneering concept, first proposed for nuclear receptors by O'Malley and colleagues in the early 1990s, is based on the observation that NR-dependent transcription can be achieved in the absence of a classical ligand by activation of cell signaling pathways at the membrane that leads to increased phosphorylation of the receptor or its associated partners (52). It is proposed that such phosphorylation events facilitate NR-coactivator interactions leading to an increased rate of transcription. For similar reasons, it can be appreciated why overexpression of either AR or its attendant cofactors can result in ligand-indepen-

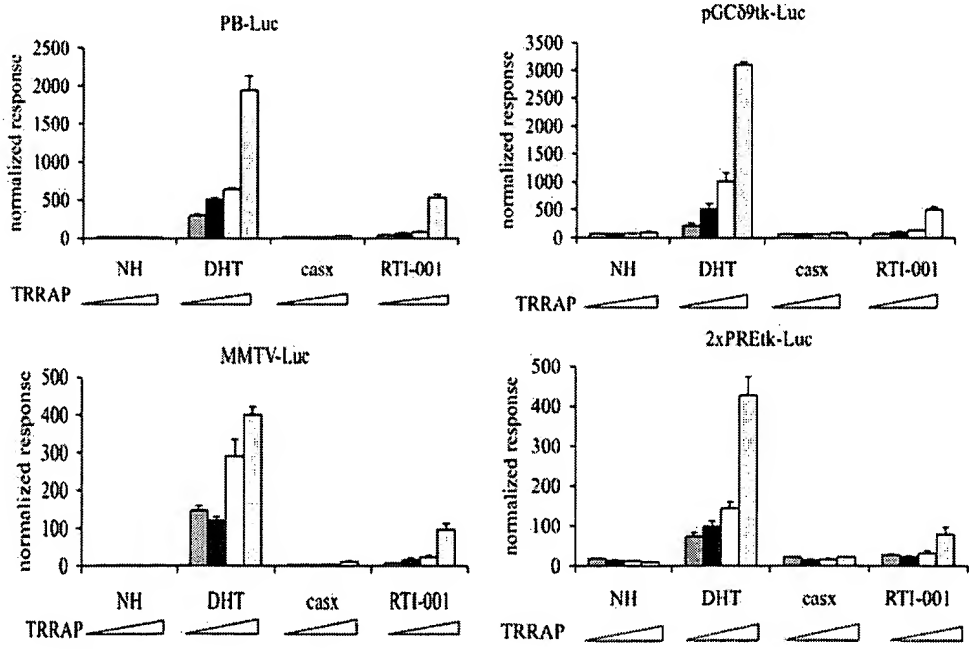


**Fig. 8.** TRRAP Functions as an AR Coactivator

CV-1 cells were transfected with increasing amounts of a plasmid expressing TRRAP (0, 135, 270, and 540 ng/well), together with SG5-AR (13.5 ng/well), different reporter constructs as indicated, and a normalization plasmid pCMVβgal (27 ng/well). Vehicle control (NH), DHT, casx, or RTI-001 were added to cells 24 h after transfection. Luciferase and β-galactosidase activities were measured 16 h after ligand addition. The values of the luciferase activity were normalized to those of the β-galactosidase and expressed as normalized response.

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dent activation. Regardless of the mechanism, it is clear that coactivator recruitment is an obligate step for either ligand-dependent or ligand-independent AR transcriptional activity and that agents that interfere with this interaction should have utility as prostate cancer therapeutics. In the current study, we have clearly demonstrated, using peptide antagonists, that inhibition of AR-transcriptional activity can be achieved by disrupting AR-coactivator interactions.

Consistent with the findings by Hur *et al.*, all of our high-affinity AR-interacting peptides are rich in phenylalanine. Unlike their study, however, we did not isolate any peptides containing the WxxLW, WxxLF, or WxxVW motifs. Because our screen was designed to identify high-affinity peptides that can function as AR antagonists, we may have missed peptides that contain the latter class of motifs. Indeed, peptides containing these tryptophan-rich motifs did not interact very well with AR when tested in a M2H assay. Of note, we found quite a number of peptides that contain an FxxYF motif and contain a hydrophobic leucine residue at the −1 position and either a leucine or tyrosine at the +6 position. It is reasonable to speculate that by screening with the full-length AR, as against the isolated LBD, that the peptides identified would have to be of higher affinity because they would be competing for occupancy of AF-2 with the amino terminal FxxLF motif. We cannot rule out the possibility that the high-affinity peptides we have identified interact with the AF-2 pocket in a manner distinct from previously identified, lower affinity, AR-interacting peptides.

We were disappointed that none of the peptides identified bind outside of the LBD, even when full-length AR was used in the screen. We considered two possible explanations: 1) the N terminus was not folded correctly under the conditions of our screen and thus was not able to recruit peptides; or 2) binding of the cofactor interface on the AR-NTD requires an extended conformation that cannot be provided by small peptides used in our screens. We believe the latter may be the case, because we have recently completed multiple screens using T7 bacteriophage cDNA expression libraries and found several AR NTD interactors under similar conditions (our unpublished results). Nonetheless, in this study we found several peptides that, when overexpressed in cells, efficiently inhibit AR-mediated transcription. We believe that these peptides have the potential to be developed into peptide antagonists of AR or can be used in compound screens to identify antagonists with similar binding characteristics that can be used to block this specific AR:cofactor interface.

Finally, the identification of TRRAP as an AR cofactor by homology searches using the sequences of the isolated peptides highlights the utility of this approach to identify potential partners of NRs *in silico*. Previously, we have had success using this approach to identify proteins that interact with ER, the nuclear receptor corepressor, and steroid receptor coactivator-3 (16, 36). Although not contemplated when we began using combinatorial peptide phage display, it has proven to be a useful companion to other technologies designed to identify NR cofactors.

With respect to therapeutic development, the validation of the AR coactivator binding pocket as a drug target is one of the most important aspects of the current study. However, this project was undertaken also as part of a larger effort, NURSA, to develop reagents, protocols, and technologies that could benefit the nuclear receptor community as a whole and that may increase the translational benefits from NR research. Thus, although those interested in AR signaling may find the specific peptide antagonists of this receptor useful in their studies we believe that the validation of the BioBac system as a means to produce full-length nuclear receptors is also likely to have a significant impact on the field. Most cofactor identification studies performed to date have used the LBD of the NR of interest as bait in protein-protein screens. However, as discussed above, we have determined, with several receptors, that the overall surface of the LBD in the context of the full-length protein and as an isolated domain is not the same. Clearly, the availability of soluble biologically active AR will enable researches to circumvent these problems. In addition to AR, we have now used this system to produce ERR$\alpha$ (ER-related receptor $\alpha$), PR, ER$\alpha$, GR, Nurr1 (nuclear receptor-related 1), CoupTF (chicken ovalbumin upstream promoter transcription factor 1), and retinoid X receptor-$\alpha$. We believe that the ease of use of this system will facilitate the progress in many aspects of NR research where purified recombinant full-length proteins are required but have been hard to produce.

## MATERIALS AND METHODS

### Production and Purification of Recombinant AR

Full-length AR cDNA was subcloned into the *ApaI* site in the baculovirus shuttle vector pDW464 (ScienceReagents, Inc., El Cajon, CA) to generate an in-frame fusion of AR with the biotin acceptor peptide. The resulting plasmid was recombined with the baculovirus genome in *E. coli* DH10Bac cells (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The recombinant baculovirus DNA was recovered from DH10Bac and used to transfect Sf9 cells to produce baculovirus and recombinant protein. Sf9 cells were maintained in serum-free SFX media [Hyclone (Logan, UT) or Invitrogen] in a shaking incubator. Log-phase Sf9 cells were infected with MOI (multiplicity of infection) = 1 of AR baculovirus, and the synthetic AR agonist R1881 (1 $\mu$M) was added to the culture 24 h after infection. Cells were pelleted after 48 h infection, washed once with ice-cold PBS, and lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 10 mM $\beta$-mercaptoethanol, 100 mM KCl, 1% Nonidet P-40, 1:200 protease inhibitor cocktail III, 1 $\mu$M R1881, 50 mM sodium fluoride, and 50 mM $\beta$-glycerophosphate]. The lysate was incubated for 1 h at 4 C with gentle rocking followed by centrifugation at 20,000 rpm for 20 min to obtain the soluble fraction. Glycerol was added to a final concentration of 20% to the soluble fraction and the lysate was stored at −80 C until ready for purification. Recombinant bT-AR was purified from soluble lysate using Softlink avidin beads (Promega Corp., Madison, WI) using either

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batch or column purification protocol. For batch purification, Softlink avidin beads were first activated according to manufacturer's protocol and then incubated with AR-containing lysate for 2 h at 4 C. The beads were washed three times with buffer A [20 mM Tris (pH 8.0), 500 mM KCl, 10 mM β-mercaptoethanol, 10% glycerol, 1 μM R1881], four times with buffer B [20 mM Tris-HCl (pH 8.0), 1 M KCl, 10 mM β-mercaptoethanol, 10% glycerol and 1 μM R1881] and once with buffer C: Tris-HCl (pH 8.0), 200 mM NaCl, 2 mM EDTA, 4 mM dithiothreitol, 10% glycerol, 1 μM R1881, 1 mM CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate). bT-AR was eluted four times, 500 μl each, with elution buffer (buffer C plus 5 mM biotin). Same buffers were used in column purification. Detailed protocols are available upon request.

### M13 Phage Panning

M13 peptide panning protocol has been described previously (28, 29). Briefly, 2 μg of purified bT-AR were added to each well of a 96-well plate in the presence of 100 mM $NaHCO_3$, and incubated overnight at 4 C. Next morning, protein-coated wells were blocked with 2% milk/PBS for 1 h at room temperature. Approximately $10^8$ plaque-forming units of phage libraries were added to each well and incubated with target protein for 3 h at room temperature. The wells were washed five times with PBST (PBS + 0.1% Tween 20) and bound phage eluted with 100 μl of 0.1 M HCl. The eluent was neutralized with 50 μl of 1 M Tris-HCl (pH 7.4) and the eluted phage was amplified in DH5αF' cells for 5 h in a 37 C shaking incubator. Amplified phage were recovered from bacterial supernatant and subjected to subsequent rounds of panning. Five rounds of panning were performed, and enrichment of target binding phage was monitored using an ELISA as described (28, 29). PCRs were used to recover peptide inserts from bacterial supernatant that showed significant enrichment of target binding phage. The PCR products were digested with *Xho*I and *Xba*I before ligation into the expression vector pMsx for mammalian two-hybrid analysis.

### Mammalian Cell Culture and Transfection

CV-1 and HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in MEM (Invitrogen) supplemented with 8% fetal bovine serum (Hyclone), nonessential amino acid and sodium pyruvate (Invitrogen). All transfections were performed using Lipofectin (Invitrogen) as described previously (29). Briefly, for mammalian two-hybrid analysis of receptor: peptide interactions HepG2 cells were split into 96-well plates a day before transfection in MEM +8% fetal bovine serum. On the day of transfection, media were removed and cells were washed once with PBS followed by addition of DNA:lipofectin mix and incubated for 5 h. The transfection was stopped by removing DNA:lipofectin mix and replacing it with fresh phenol red-free MEM + 8% charcoal-stripped serum (Hyclone). A saturating concentration of hormone (100 nM) or vehicle control (100% ethanol) were added to the cells 24 h later, and the luciferase and β-galactosidase assays were performed 16–18 h after hormone addition. The following DNA concentrations were used in the mammalian two-hybrid transfections: 54 ng/well of 5xGal4-Luc3 reporter, 5.4 ng/well pCMVβgal, 21.6 ng/well pM-peptide and different amounts of VP16-receptor constructs to adjust for the differences in receptor expression levels. Amounts of herpes simplex virus VP16 transactivator protein (VP16)-receptor used: VP16-ERα: 81 ng/well, VP16-PRB: 21.6 ng/well, VP16-GR: 54 ng/well, VP16-AR: 2.16 ng/well. Transfections receiving less VP16-receptor DNA were adjusted with empty VP16 to a total of 81 ng/well. Approximately equal expression of receptors was verified by Western blot analysis using an anti-VP16 antibody (Sigma, St. Louis, MO).

### Western Blot Analysis

CV-1 cells transfected with various constructs were lysed directly in 2× SDS-PAGE sample buffer and boiled for 5 min at 95 C. Proteins were resolved on SDS-polyacrylamide gels and transferred to nitrocellulose membrane (Amersham Corp., Arlington Heights, IL) Expression of peptides was detected with an anti-Gal4DBD antibody (1:500 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Anti-AR441 antibody [1:1000 dilution, D. Edwards and N. Weigel, University of Colorado (Denver, CO) and Baylor College of Medicine (Houston, TX), respectively] was used to detect AR expression. The expression of VP16-receptor fusion proteins were detected using anti-VP16 antibody (1:1000 dilution, Sigma).

## Acknowledgments

We thank Dr. D. Kenan (Duke University, Durham, NC) for providing M13 peptide libraries, Dr. P. Turnbull (GlaxoSmith-Kline, Research Triangle Park, NC) for bicalutamide and Dr. E. Cook (Research Triangle Institute, Research Triangle Park, NC) for the RTI compounds. We also thank Drs. D. Edwards (University of Colorado, Denver, CO) and N. Weigel (Baylor College of Medicine, Houston, TX) for their generous gift of anti-AR441 antibody, Drs. Livingston and Man (Dana-Farber Cancer Institute, Boston, MA) for the TRRAP expression plasmid.

Received February 3, 2005. Accepted March 23, 2005.

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This work was supported by Nuclear Receptor Signaling Atlas (NURSA) Grants U19 DK062434 and CA95094 (to C.Y.C.) and DK-065251 (to D.M.D.).

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